ANTIVIRAL ACTIVITY AND CHEMICAL COMPOSITION OF EUROPEAN AND EGYPTIAN PROPOLIS

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Propolis, GC/MS, Polyphenols, Antimicrobial Activity

Four propolis samples from Austria, Egypt, France and Germany were investigated by GC/MS, where twenty compounds were being new for propolis. The samples showed some similarities in their qualitative composition. phenylethyl-trans-caffeate, benzyl ferulate and galangin were predominant in German propolis. Benzyl caffeate was predominant in French sample. pinocembrin was predominant in French and Austrian propolis and trans-p-coumaric acid was predominant in all samples. Egyptian propolis is characterized by the presence of unusual esters of caffeic acid with C12- C16 fatty alcohols, mainly saturated.

The antiviral activity of propolis samples from Austria, Egypt, France and Germany against avian reo virus (ARV) and infectious bursal disease virus (IBDV) was evaluated. All propolis samples reduced the viral infectivity in varied degree according to the propolis origin. Egyptian propolis showed the highest antiviral activity against avian reo virus and infectious bursal disease virus.

Introduction

Propolis (bee glue) is a resinous hive product. It consists of exudate from plants mixed with beeswax and used by bees as glue for general-purpose, sealer and draught-excluder for beehives. It was used for a long time as early as 3000 BC (Hegazi, 1998). Propolis possesses variable biological activities: antiviral activity against Newcastle Disease Virus (Hegazi et al., 1993); Rift Valley Fever Virus (Hegazi et al., 1997), anti-small pox (Krivoruchko et al. 1975); anti-influenza virus (Manolova et al., l985); antibacterial (Tothne, 1987; Hegazi et al., 1996-a); fungicidal (Millet et al., 1987, Hegazi et al., 1996-b and Hegazi et al., 2000); antiulcer and anti-tumour etc. (Marcucci , 1995; Cheng & Wong, 1996).

Infectious bursal disease virus (IBDV) is a non-enveloped virus of double stranded RNA type which belongs to Birnaviridae family (Brown, 1986). This virus replicates in chicken macrophages and lymphoid cells of Bursa of Fabricius (Cheville, 1967) which lead to immunosuppression (Hegazi and Shalaby 1994). Immunosuppression makes birds very susceptible to pathogenic and many otherwise non-pathogenic organisms (Shome et al., 1997).

Avian reo virus (ARV) is worldwide distribution, occurring in all major poultry farms. The incidence is high essentially in the early stage of life (Robertson & Wilcox, 1986 and Hegazi et al., 1989)).

The chemical composition of propolis appeared to be extremely complex and more than 180 compounds have been identified so far (Marcucci, 1995), the most important ones being polyphenols. In temperate climatic zones (Europe, North America, Mongolia, Uruguay, New Zealand) the main source of propolis is poplar buds, mainly these of Populus nigra (Greenaway et al., 1987 ; Wollenweber et al., 1987 ; Bankova et al., 1992 ; Bonvehi et al., 1994 ; Markham et al., 1996), but in some cases other poplar species can be used as an additional supply of propolis (Greenaway et al.,1989; 1990a; 1990b). In such cases chemical composition of propolis which is connected with its biological activity will be changed.

The aim of the present communication was to study the antiviral activity of propolis from different countries against ARV and IBDV.

Materials and Methods

Propolis
Propolis samples were collected in Austria (Vienna), Egypt (Bani Sweif), France (Monbliahue) and Germany (Hannover).

Extraction and sample preparation
One gram of each sample was cut into small pieces and extracted at room temperature with 50 ml of 70% ethanol (twice after 24 hours). The alcoholic extract was evaporated under vacuum at 50 °C till dryness. 2.5 mg of the dried matter was prepared for chromatography according to Greenaway et al. (1990),and Hegazi et al. (2000).

Antiviral activity

Viral titration
The viral activity of ARV and IBDV viruses was determined to evaluate the infectivity titter in chicken fibroblast cell cultures. Embryonated eggs were obtained from the Faculty of Agriculture, Cairo University. Primary monolayer cell cultures of 9 to11 day old chick embryo fibroblast (CEF) were prepared in plastic plates (Falcon 3002, Becton Oxnard, Calif). CEF fibroblasts grown in microtiter plates were used for virus titration by incoluated each virus dilutions in 5 wells of CEF fibroblast cultures. IBDV was a local isolates from Animal Health Research Institute, Dokki, Giza, Egypt. ARV was vaccinal strain S 1133 was kindly supplied by Animal Health Research Institute, Dokki , Egypt.

The tissue culture infectivity dose that causes cytopathogenentic effect in 50 % of the cell culture ($TCID_{50}$) was calculated.

Antiviral activity of propolis
The ethanolic extract from one gram raw propolis was variable in weight (Austrian propolis 0.352 gm/dry weight, Egyptian propolis 0.234 gm/dry weight, French propolis 0.20 gm/dry weight and German propolis 0.187 gm/dry weight) dissolved in phosphate buffer saline (PBS, pH 7.2) to obtain a 1 % stock solution. Titration of antiviral activity was done by mixing equal volume of serial ten fold dilutions of each virus with 1 % stock solution of propolis The mixture were incubated at 37 ^{o}C for incubated for 30 minutes. Following this step 50 μl of each mixtures were inoculated into CEF cell cultures using 5 well / each mixtures. Back titration of ARV (Taylor et al., 1966) and IBDV (Komine et al., 1989) was done, using 5 wells / virus dilution; also 5 well were used for propolis control (to test for cell cytotoxicty) and 5 wells were left as cell control. After 120 hours, cells were observed microscopically for cytopathic effects. Cell monolayers were stained with crystal violet. The effect of propolis on ARV and IBDV infectivity mean tissue culture infective dose ($TCID_{50}$) was calculated according to Reed and Muench (1938).

GC/MS analyses

A finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-1 column, 30 m x 0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure , 20 kg/cm2 ; injector temperature, 310ºC; GC temperature program, 85-310ºC at 3ºC / min.(10-min. initial hold) . The mass spectra were recorded in electron ionization (EI) mode at 70 eV. The scan repetition rate was 0.5 s over a mass range of 39 –650 atomic mass units (AMU).

Identification of compounds

The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed where possible to confirm GC retention times.

Results and Discussion

Propolis samples from Austria, France, Egypt and Germany has been investigated. The percentage of extracted matter was as follows: Austrian propolis 0.352 gm/dry weight, Egyptian propolis 0.234 gm/dry weight, French propolis 0.20 gm/dry weight and German propolis 0.187 gm/dry weight. The alcoholic extracts (extract with 70% ethanol) were subjected to preliminary investigation by thin layer chromatography (TLC) and the spots of flavonoids and phenolic esters showed some similarities in the 4 samples. The samples were silylated and subjected to GC/MS analysis. The results obtained are summarized in Table I.

It is evident that the all propolis samples showed significant qualitative similarities. The quantitative differences obtained could be due to the participation of different poplar species. The aromatic acids, found in all investigated samples as caffeic acid which has the largest quantity in all sample. The following acids were identified for the first time in propolis: 2-Phenyl-2-hydroxyacrylic acid identified in Austrian and German samples, 4-methylmandelic acid identified in Austrian, German and French samples. Beside aromatic acids poplar pris characterized by the presence of significant amounts of esters of these acids. Phenylethyl caffeate appeared in all samples. Benzyl-trans-4-coumarate, cinnamyl-trans-4-coumarate, cinnamyl isoferulate, 3-methyl-3-butenyl trans-caffeate, 3-methyl-2-butenyl trans-caffeate and cinnamyl caffeate were found in different concentrations in German, Austrian and French propolis. Prenylated caffaete esters were present in all samples. Benzyl caffeate present also in all samples with higher concentration in French propolis. Besides the known esters propolis contains three new ones: benzene propanoic acid ethyl ester in French propolis, butanyl-caffeate and phenylethyl- caffeate isomer in German propolis. Egyptian propolis is characterized by the presence of unusual esters of caffeic acid with C12- C16 fatty alcohols, mainly saturated. These esters have not been found till now in propolis. Flavonoid aglycones, especially flavanones are typical components of poplar propolis. All investigated samples contain significant amount of flavanones, but there are differences in the concentration of the individual compounds. Pinocembrin is the main flavanone of the all samples. German propolis is characterized by the presence of significant amounts of galangin. Between the main components of the samples investigated appeared to be Hydroxyacetic acid and 5-hydroxy-n-valeric acid, 2,3-butanediol, guiacol and methylglucose which were identified for the first time in propolis. A series of triterpenes in Egyptian propolis, including the characteristic animal sterol precursor lanosterol was also identified.

The composition of propolis is therefore directly related to the composition of the poplar bud exudate collected by the bees. There are a considerable difference in bud exudate composition between different poplar species (Papay et al., 1985, 1987; Bankova et al.1989, 1994; Wollenweber et al., 1987Greenaway et al., 1989, 1990a, 1990b, 1990c and Hegazi et al., 2000). A comparative GC/MS investigation of different propolis samples from 4 countries. Was found 87 compounds have been identified, which included 20 identified for the first time in propolis. The relative concentration of these compounds varies greatly and these variations are species-specific. Wollenweber (1975) reported that some Populus spp., such as aspens, are characteristically high in cinnamic acid derivatives and low in flavonoids. Phenylethyl-trans-caffeate isomer, pinokansin acetate and galangin were predominant in German propolis (Hegazi et al., 2000). Pinocembrin was predominant in French and Austrian propolis and trans-p-coumaric acid was predominant in German, Austrian, and French. A series of triterpenes in Egyptian propolis, including the characteristic animal sterol precursor lanosterol was also identified (Bankova et al., 1997 and Christove et al., 1998).

Virus titration

The IBDV stock infectivity titer was $10^{9.85}$ $TCID_{50}$ /ml and ARV stock infectivity titer was $10^{8.65}$ $TCID_{50}$ /ml.

Biosafety of propolis in chicken embryo fibroblast cell culture

The biosafety of propolis in chicken embryo fibroblast cell culture was done . The propolis samples investigated were non cytotoxic to CEF cell cultures

The effect of propolis on the infectivity titers as measured by the mean tissue culture infective dose ($TCID_{50}$) of ARV and IBDV viruses was illustrated in Table 2. It was clear that the dilution of propolis (1/100) of the original dilution (100 mg / ml) of all propolis samples from different countries revealed reduction in the infectivity mean titers of ARV. It was obvious that the reduction varied from propolis sample to the another. Egyptian propolis reduced ARV infectivity by 3.04 $\log_{10}$; in German 4.05 $\log_{10}$; in Austrian propolis 5.02 $\log_{10}$ and French propolis 6.03 $\log_{10}$ if compared with the virus control 8.65 $\log_{10}$.

The influence of propolis on IBDV virus was demonstrated in Table (2). It was clear that the dilution of propolis (1/100) of the original dilution (100 mg / ml) of all propolis samples from different countries revealed reduction in the infectivity mean titers of IBDV. It was obvious that the reduction varied from propolis sample to the another. Egyptian propolis reduced IBDV infectivity by 2.04 $\log_{10}$; in German 4.22 $\log_{10}$; in Austrian propolis 3.01 $\log_{10}$ and French propolis 2.53 $\log_{10}$ if compared with the virus control 9.85 $\log_{10}$.

The influence of propolis on reproduction of ARV and IBDV viruses was manifested by reduction in infectivity titer as calculated by the mean tissue culture infective dose ($TCID_{50}$) of ARV and IBDV. Similar results were observed by Maolova et al., (1985) who found an inhibitory effect of influenza virus by different fractions of propolis. Krivoruchko et al., (1975) Found that a sharp reduction of the ineffectiveness of small pox vaccine virus within 15 minutes at 20°C on using an aqueous extract of propolis in vitro. Also Hegazi et al., (1993) studied the effect of propolis on different NDV vaccinal strains. It was clear that the addition of propolis to NDV induced a significant reduction of infectivity titers. The effect of propolis was pronounced in Lasota, Clone 30 and virulent NDV. The HA titers of Komarov and virulent virus were reduced significantly. Kujumgiev et al., (1999) investigated propolis samples from different geographic origins for their antiviral (against avian influenza virus) activity. Also Hegazi et al., (1997) found that the aqueous extract of propolis and honey reduced the morbidity and mortality rates as well as decreased the infectivity mean titer of Rift valley fever virus (RFV) infected baby mice. The infectivity of both viruses was reduced, but this reduction was varied according to the propolis origin. The reduction of the infectivity depends on the chemical composition of different propolis collected from different countries. These findings of the differences of chemical composition were previously reported as considerable differences in bud exudate composition between different poplar species (Papay et al., 1985, 1987; Bankova et al.1989, 1994; Wollenweber et al., 1987; Greenaway et al., 1989, 1990a, 1990b, 1990c and Hegazi et al., 2000). The activities of all samples were similar in spite of the differences in their chemical composition. It is evident that the all propolis samples showed significant qualitative similarities. The quantitative antiviral activity differences obtained could be due to the participation of different poplar species (Hegazi et al., 2000).

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Table 1: Chemical composition assessed by GC/MS of alcohol extracts of different propolis samples.

Compound	Austria	Germany	France	Egyptian
	%TICa			
Acids (aliphatics)				
Lactic acid	0.30	0.23	0.50	1.3
Hydroxyacetic acid [b]	0.20	0.05	0.40	
5-Hydroxy-n-valeric acid [b]	0.10	0.04	0.10	
2,3-Dihydroxypropanoic acid		0.03		
Nonanoic acid		0.03		
Malic acid		0.02	0.20	
Dodecanoic acid	0.10	0.05	0.20	
Tetradecanoic acid			0.10	
Palmitic acid	1.80	3.20	3.00	3.0
Linoleic acid	0.70	0.06	0.70	
Oleic acid	1.00	1.00	2.40	4.0
Stearic acid	1.00	3.40	1.00	0.9
Eicosenoic acid			0.50	
Eicosanoic acid			0.20	
Docosanoic acid			2.00	
Tetracosenoic acid	1.20	3.00	1.60	1.6
Hexacosanoic acid		050		
Succinic acid				0.3
Piruvic acid b				1.3
Acids (aromatics)				
Benzoic acid	3.1	1.30	4.0	0.2
Hydrocinnamic acid	0.3	0.10	0.5	
Trans- Cinnamic acid	4.8	0.40	2.0	
2-Phenyl- 2-hydroxyacrylic acid [b]	.03	0.05		
4-Methylmandelic acid b	0.3	0.05	0.3	
4-Hydroxy benzoic acid	0.3	0.06	0.4	
4-Methoxyhydrocinnamic acid	0.3		0.2	
3-Hydroxybenzeneacetic acid [b]			0.2	
cis-P-Coumaric acid	0.3		0.2	
4- Methoxycinnamic acid	0.2	0.30	0.5	
trans- P-Coumaric acid	7.0	6.70	6.1	0.5
3,4-Dimethoxy-cinnamic acid		0.23	2.2	0.4
Isoferulic acid	1.3	1.80	2.3	
Ferulic acid	2.6	0.05	2.1	0.2
Cffeic acid	2.6	2.60	5.2	0.3
Esters				
Benzenepropanoic acid ethyl ester [b]			0.1	
Benzylybenzoate	0.9	0.03	0.2	
Cinnamyl cinnamate	0.2	0.70	0.2	
Pentenyl coumarate	0.1	0.30		
Benzyl-trans-4- coumarate	3.5	2.70	4.0	
Phenyl-ethyl-trans- coumarate	0.3	0.40	0.3	
Cinnamyl- trans- coumarate	1.1	3.40	2.1	
3-Methyl-3-butenyl-isoferulate	0.5	0.60	0.6	
3-Methyl-2-butenyl-isoferulate	1.0	0.70		
Cinnamyl –isoferulate		2.80	1.3	
Benzyl –ferulate			7.3	
Cinnamyl –ferulate		0.20		
Ethyl caffeate		0.30		
Butanyl-caffeate [b]		0.08		
3-Methyl-3-butenyl- caffeate	2.7	2.40	2.7	0.9
3-Methyl-2-butenyl- caffeate	3.0	2.40	2.4	1.3
Benzyl – caffeate	3.1	1.50	14.5	0.6
Phenyl-ethyl – caffeate	2.4	5.80	5.1	

Compound	Austria	Germany	France	Egyptian
Phenyl-ethyl – caffeate (isomer) [b]		17.00		
Cinnamyl – caffeate	0.6	5.60	3.1	
Dodecyl caffeate [b]				1.1
etradecyl caffeate [b]				3.1
Tetradecenyl caffeate [b]				0.3
Hexadecyl caffeate [b]				4.7
Diterpe and triterpinoids				
Dehydroabietic acid	0.2	0.08	0.05	
lanosterol				1.2
cycloartenol [b]				7.1
triterpenic alcohol of amyrine type [b]				4.8
ß-amyrine [b]				4.7
Flavonoids				
2`,6`-Dihydroxy-4`-methoxy dihydrochalcone			0.2	
Pinostrobin chalcone				
Pinostrobin	0.6	1.40	1.2	0.6
Pinocembrin	15.3	6.90	17.2	1.1
Pinobankasin	2.7	4.80	4.8	0.3
Pinobankasin-3-acetate	6.1	9.30	9.0	1.1
Chrysin	2.5	3.50	5.3	0.8
Galangin	6.4	21.60	10.0	0.7
5,7- Dihydroxy-3-butanoyloxy flavanone	1.0	3.00	1.3	
5,7- Dihydroxy-3-pentenoyloxy flavanone [b]		0.50		
5,7- Dihydroxy-3-pentanoyloxy flavanone		0.20		
Sugars				
2,3,5,6,Tetrahydroxy-methylglucofuranoside [b]	0.5	1.60	0.6	
Fructose	3.2	6.10	3.0	3.1
Sorbose	3.0	3.50	2.8	3.1
Galactose		0.50		
Glucose	2.2	8.80	1.0	6.1
Sugar (unidentified)		0.40		
Glucose dimer		13.20		
Sucrose				1.6
Mannitol				0.2
Others				
Phosphoric acid [b]	0.2	0.10	0.3	2.7
Glycerol octadecyl ether				1.8
2,3-butanediol [b]		0.4	0.5	
Guiacol [b]	2.3	0.23	1.4	

a The ion current generated depends on the characteristics of the compound concerned and it is not true quantitation.
b For the first time in propolis.

Table 2
Effect of propolis on virus infectivity mean titer

Sample	Virus log 10			
	Reo		IBDV	
	Reo	Reo + P	IBDV	IBDV+ P
Austrian	8.65 *	5.02	9.85	3.01
Egyptian	8.65	3.04	9.85	2.04
French	8.65	6.03	9.85	2.53
German	8.65	4.05	9.85	4.22

P = 1 % Propolis extract.
* = Calculated of their infectivity as the mean tissue culture infective dose (TCID50).